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REPORT NUMBER THREE

# GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1982

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. Attention was concentrated on the following areas during the past year: (1) The influence of the B. anthracis plasmid, pBA1, on the physiology of the organism, and (2) Genetic exchange

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20. Abstract (Continued)

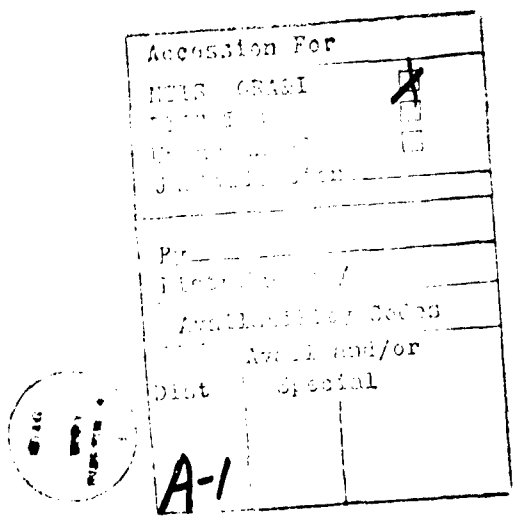
systems for B. anthracis.

Cells harboring pBA1 and those cured of the plasmid differ in the following aspects. (1) Cured cells do not produce detectable amounts of protective antigen in the Casamino acids medium used routinely for protective antigen production; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells sporulate earlier and at a higher frequency than uncured cells; (4) Spores from cured cells are more heat resistant than spores from uncured cells; (5) Cured cells are more sensitive than uncured cells to bacteriophages CP-2, CP-20, and CP-51; and (6) Cured cells have altered growth characteristics in synthetic media.

Although cured strains grow more poorly in synthetic media than their uncured parental strains, experimental results suggest it is unlikely that loss of the plasmid results in acquisition of specific growth requirements. It seems more likely that loss of the plasmid affects regulatory or transport activities of the cell.

Although phage CP-51 is very effective in transferring small plasmids among strains of B. anthracis, B. cereus, and B. thuringiensis, attempts to demonstrate transfer of pBA1 by the phage have been unsuccessful. pBA1 is probably too large to be packaged by CP-51.

The problem in transformation of B. anthracis protoplasts lies in the difficulty in regenerating protoplasts into cells following exposure to DNA. Recently regeneration of protoplasts produced by a new procedure has been possible and transformation with small plasmid DNA at a very low frequency has been demonstrated.



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## SUMMARY

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The toxin-producing but avirulent Weybridge (Sterne) strain of B. anthracis is being used in all our studies. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. During the past year our major effort was placed on two areas of research: (1) the influence of the B. anthracis plasmid, pBA1, on the physiology of the organism, and (2) genetic exchange systems for B. anthracis.

We have observed the following differences between cells carrying pBA1 and those that have been cured of the plasmid. (1) Cured cells do not produce detectable amounts of protective antigen in the Casamino acids medium used routinely for protective antigen production in our laboratory; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells sporulate earlier and at a higher frequency than uncured cells; (4) Spores from cured cells are more heat resistant than spores from uncured cells; (5) Cured cells are more sensitive than uncured cells to bacteriophages CP-2, CP-20, and CP-51; and (6) Cured cells have altered growth characteristics in synthetic media.

Advantage was taken of the observation that strains cured of pBA1 sporulate earlier and at a higher frequency than strains harboring the plasmid to develop an improved method for isolating cured strains. The fact that colonies of cured and uncured cells differ in morphology serves as the basis for tentative identification of cured strains. A number of cured derivatives have been isolated from several genetically marked strains (auxotrophic mutants). All cured strains we have tested are similar with respect to the characteristics listed above.

The difference in colonial morphology of cured and uncured strains is probably a reflection of their sporulation characteristics. In broth, as well as on agar medium, cured cultures sporulated earlier and to a greater extent than uncured cultures. We were surprised to find that spores of cured strains were considerably more heat resistant, than spores of uncured strains.

Cured cells were more sensitive than uncured cells to three different bacteriophages. Preliminary experiments suggest that the difference in phage sensitivity is not a reflection of a restriction-modification system or a difference in kinetics of phage adsorption.

Cured strains grew more poorly in synthetic media than their uncured parental strains. However, it seems unlikely that loss of the plasmid results in acquisition of specific growth requirements. It seems more likely that loss of the plasmid affects regulatory activities or perhaps transport activities of the cell. Consistent with this idea is the fact that growth of cured strains in synthetic media was enhanced significantly by decreasing the concentration of phosphate; uncured strains, however, grew better with higher concentrations of phosphate.

Although phage CP-51 is very effective in transferring small plasmids among strains of B. anthracis, B. cereus, and B. thuringiensis, we have been unsuccessful in our attempts to demonstrate transfer of the B. anthracis plasmid, pBA1, with CP-51. pBA1 is probably too large to be packaged by CP-51. For that reason we will continue to look for host-range mutants of TP-12 and TP-13, transducing phages for B. thuringiensis which are large enough to package a plasmid the size of pBA1.

Until very recently we have been unable to demonstrate any transformation of B. anthracis. We have not been able to induce competence for transformation in intact cells. The problem in transformation of B. anthracis protoplasts lies in the difficulty of regenerating protoplasts into cells following exposure to DNA. Recently we have been able to regenerate protoplasts obtained by a new procedure and we have, in fact, obtained transformation with plasmid pUB110 DNA at a very low frequency. We are now concentrating on trying to improve the transformation procedure.

## Foreward

Citations of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such items.

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This is the third annual report submitted under contract No. DAMD 17-80-C-0099. Research on the contract began July 1, 1980. The contract was extended for a second year beginning July 1, 1981 and on July 1, 1982 it was extended a second time for a period of two years. The first and second annual reports are dated December 1980 and December 1981, respectively.

During the year represented by this annual report our research concentrated largely on the influence of the B. anthracis plasmid on the physiology of the organism. Considerable effort was also applied toward development of genetic exchange systems for B. anthracis. In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections. Abstracts of two papers which arose from the work on this contract and which will be presented at the annual meeting of the American Society for Microbiology in March 1983 are also included.

In this and future reports and publications I will refer to the plasmid associated with toxin production by the Weybridge strain of B. anthracis as pBA1. Similar plasmids in other strains of B. anthracis will presumably be assigned different and specific designations by investigators as appropriate. I suggest that the designations pBA2, pBA3, etc. be used for similar plasmids in other strains. I suggest further that my laboratory be used as a clearing center for assigning designations to B. anthracis plasmids.

## MATERIALS AND METHODS

Organisms. The Weybridge (1) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England in 1957. It was isolated by Sterne (2) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Table 1 lists specific strains and mutants referred to in this report.

Media. For convenience to the reader compositions of the various media mentioned in this report are given below in detail. All amounts are for one liter final volume. For preparations of solid medium, 15 grams of Difco agar were added per liter of the corresponding broth.

NBY broth: Difco nutrient broth, 8 g; Difco yeast extract, 3 g.

PA (phage assay) broth: Difco nutrient broth, 8 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; pH adjusted to 6.0 with HCl.

L broth: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g. pH adjusted to 7.0 with NaOH.

BHI broth: Difco brain heart infusion broth, 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I: $(NH_4)_2SO_4$, 2 g; $KH_2PO_4$, 6 g; $K_2HPO_4$, 14 g; Sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.04 g; $MnSO_4 \cdot H_2O$, 0.00025 g; pH adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ are sterilized separately.

Minimal IC: Minimal I with 5 g of Difco vitamin-free Casamino acids and 10 mg of thiamine hydrochloride.

Minimal M: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Antisera: All antisera were kindly supplied by Dr. Anna Johnson of USAMRIID.

Antigen assays: These were carried out by the agar diffusion method as outlined by Thorne and Belton (1).

Phage CP-51 propagation and assay: The methods described by Thorne (3) were followed. The indicator for routine assay of CP-51 was *B. cereus* NRRL B-569.

Table 1

List of strains and mutants specifically referred to in this report

| Organism | Characteristics and Source* |
|---|---|
| *Bacillus anthracis* | |
| Weybridge | Avirulent Sterne-type strain, originally obtained from MRE, Proton, England |
| Weybridge A | Colonial variant isolated from Weybridge (Thorne) |
| Weybridge B | Colonial variant isolated from Weybridge (Thorne) |
| Weybridge A M2 | $Trp^-$ mutant of Weybridge A |
| Weybridge A M14 | $Phe^-$ mutant of Weybridge A |
| Weybridge A M18 | *pyrA* mutant of Weybridge A |
| Weybridge A M23 | $Ura^-$ mutant of Weybridge A |
| Weybridge M44 | $Trp^-$ mutant isolated from wild-type Weybridge strain |
| Weybridge A M2 tdl | $Trp^-$, $Tet^R$, (pBC16) by transduction with CP-51 |
| Weybridge A M18 tdl | *pyrA*, $Tet^R$, (pBC16) by transduction with CP-51 |
| Weybridge A M18 tdl cured 28 | *pyrA*, $Tet^R$, $(pBA1)^-$ derivative of A M18 tdl |
| Weybridge A M18 tdl cured 39 | *pyrA*, $Tet^S$, $(pBA1)^-$ and $(pBC16)^-$ derivative of A M18 tdl |
| Weybridge A M23 cured 1 | $Ura^-$, $(pBA1)^-$ derivative of A M23 |
| *Bacillus cereus* | |
| NRRL B-569 | U.S.D.A. Laboratory, Peoria, Illinois |
| ATCC 6464 | ATCC, carries phage CP-53 |
| 6464C | ATCC 6464 cured of phage CP-53 |
| GP7 | Carries pBC16. Bernhard, et al. (9) |
| *Bacillus thuringiensis* | |
| NRRL B-4040 | subsp. *finitimus*, U.S.D.A. Laboratory, Peoria, Illinois |
| NRRL B-4041 | subsp. *alesti*, U.S.D.A. |
| NRRL B-4060 | subsp. *thompsoni*, U.S.D.A. |
| 4060C | Colonial variant of 4060 (Thorne) |

Table 1 Continued

List of strains and mutants specifically referred to in this report

| Organism | Characteristics and Source* |
|---|---|
| Bacillus licheniformis | |
| ATCC 9945A | C.B. Thorne collection |
| Bacillus subtilis | |
| 168 | trpC, C.B. Thorne collection |

*All auxotrophic mutations in the Weybridge strain were induced with UV light. All the Weybridge mutants carry pBA1 unless specifically designated $(pBA1)^-$.

Procedure for preparing Casamino acids medium for protective antigen production:

| | Stock solution mg/100 ml | Ml of stock solution per liter of medium | Final concentration (mg/liter) |
|---|---|---|---|
| $CaCl_2 \cdot 2H_2O$ | 147 | 10 | 14.7 |
| $MgSO_4 \cdot 7H_2O$ | 100 | 10 | 10 |
| $MnSO_4 \cdot H_2O$ | 8.5 | 10 | 0.85 |
| $KH_2PO_4$ | 6800 | 10 | 680 |
| $K_2HPO_4$ | 8710 | 10 | 871 |
| $FeSO_4 \cdot 7H_2O$ | 270 | 1 | 2.7 |
| Thiamine·HCl | 1000 | 1 | 10 |
| Adenine | 21 | 10 | 2.1 |
| Uracil | 14 | 10 | 1.4 |
| L-tryptophan | 520 | 10 | 52 |
| L-cystine | 120 | 10 | 12 |
| Glycine | 150 | 10 | 15 |

Combine stock solutions as indicated and add 3.6 grams of Difco Casamino acids. Adjust pH to 6.9 and add water to a final volume of 920 ml. Dispense 92 ml into 250-ml Erlenmeyer flasks (or 460 ml into 2800-ml Fernbach flasks) and autoclave 15 minutes. Just before inoculating add 1 ml of 20% glucose (to give final concentration of 0.2%) and 8.8 ml of 9% $NaHCO_3$ (to give final concentration of 0.8%) for each 100 ml of medium.

The addition of Norit A usually increases the yield of protective antigen. The optimum amount varies with batches of Norit. Currently to each flask of medium (100 ml) we add 1 ml of a 2% suspension of Norit A. It can be added either before or after the medium is sterilized.

(footnote continued on next page)

With the higher $NaHCO_3$ concentration more consistent results are obtained with vegetative inoculum than with spores. We grow an overnight culture (shaken at 37°C) in the same medium but without $NaHCO_3$ and use 0.1 ml per 100 ml of medium. Cotton-plugged flasks of production medium are incubated statically at 37°C.

## RESULTS AND DISCUSSION

### I. Influence of plasmid pBA1 on the physiology of the Weybridge strain.

We have observed the following differences between cells carrying pBA1 and those that have been cured of the plasmid. (1) Cured cells do not produce detectable amounts of protective antigen in the Casamino acids medium used routinely for protective antigen production in our laboratory; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells sporulate earlier and at a higher frequency than uncured cells; (4) Spores from cured cells are more heat resistant than spores from uncured cells; (5) Cured cells are more sensitive than uncured cells to bacteriophages CP-2, CP-20, and CP-51; and (6) Cured cells have altered growth characteristics in synthetic media. Each of these is discussed below following the description of our method, based on some of these observations, for isolating strains cured of pBA1.

#### A. Procedure for isolating strains cured of pBA1

Our first cured strains were isolated following treatment of cultures with a combination of plasmid-curing agents as described in the Annual Report dated December 1981. Our improved current method is based on the finding that cells cured of pBA1 sporulate earlier and at a higher frequency than uncured cells. The fact that colonies of cured cells have a morphology different from that of uncured colonies serves as the basis for tentative identification of cured strains.

Details of the procedure are as follows. Auxotrophic mutants were used in these studies so that cured strains could be positively identified as having originated from the starting culture rather than from possible contaminating cells. Spores or broth cultures were streaked on L agar to produce well-isolated colonies and the plates were incubated at 37°C for 16 to 24 hours. Colonies were transferred with toothpicks to plates of Minimal IC supplemented as necessary with growth requirements of particular mutants. The plates were incubated at 43°C for 18 to 24 hours and growth from

edges of colonies was transferred to fresh plates of the same medium. These plates were then incubated at 43°C for 48 to 72 hours.

Individual colonies were suspended in 1 ml of water and the suspensions were held at 65°C for 30 mintues to kill vegetative cells. Samples of each heat-treated colony were then plated on L agar and incubated at 37°C to produce isolated colonies. It was observed that very few spores were present in most of the heat-treated suspensions of colonies. Samples of 0.1 ml yielded from 0 to 25 colonies.

The characteristic morphology of cured colonies could best be observed when colonies grown from the heat-treated suspensions were transferred with toothpicks to L agar plates and incubated at 37°C for 24 to 48 hours. The difference in appearance was most striking when both types of colonies were on the same agar plate.

The proportion of heat-treated colonies that yielded cured strains varied from experiment to experiment and possibly also among different auxotrophic mutants. However, not enough tests were done to determine whether the variation in frequency obtained with different auxotrophic mutants was in fact a reflection of differences in the mutants or whether it was merely a result of uncontrolled experimental variation. In addition to the variation in the proportion of heat-treated colonies that yielded cured strains there was also a large variation in the frequencies of cured spores among the total spores from a given colony. In some instances nearly every heat-treated colony yielded some cured cells, and in such cases the proportion of cured spores in a given suspension was usually 50 to 100 percent.

By this procedure we have isolated cured strains derived from Weybridge M44 Trp$^-$, Weybridge A M18 pyrA, A M14 Phe$^-$, and A M23 Ura$^-$. Cured strains derived from different heat-treated colonies as described above represent independent curing events. However, there is no way of knowing whether cured strains derived from the same heat-treated colony represent one or multiple curing events. For that reason we have maintained stocks of only those known to have arisen from independent curing events.

From Weybridge A M18 tdl, which also carries the tetracycline resistance plasmid, pBC16 (9), we isolated 16 independently cured strains. One of these, M18 tdl cured 39, was also found to be cured of pBC16. All the others retained pBC16. In addition to the 16 independently cured strains derived from Weybridge A M18 tdl, we have isolated 12 independently

cured strains of Weybridge A M23 Ura$^-$, one cured strain of Weybridge M44 Trp$^-$, and 2 independently cured strains of Weybridge A M14 Phe$^-$.

B. Cured strains do not produce detectable amounts of toxin components

Results of our tests on toxin production by cured strains parallel those reported by Mikesell, et al. (4). Our tests were done with the Casamino acids medium described under Materials and Methods. In that medium uncured strains produced protective antigen titers of 16 to 24 as determined by agar diffusion with burro antiserum (prepared by injection of viable Sterne spores). Cured strains, however, did not produce detectable amounts of protective antigen. Even when filtrates from cured cultures were concentrated 10-fold by freeze drying, protective antigen could not be detected. Similar results were obtained when we screened cured strains for production of lethal factor and edema factor; none was detected by agar diffusion even in concentrated filtrates. In these tests in addition to the burro antiserum we also used antiserum prepared in a goat against purified protective antigen and antiserum prepared in a rabbit against lethal factor. Concentrated filtrates of cured strains did sometimes give a diffuse line of precipitate with one or more of the antisera but the line could not be identified as one formed by a component of the toxin. Similarly, colonies of cured cells on Casamino acids medium solidified with agarose produced lines of precipitation against burro antiserum, but they could not be identified as toxin components.

C. Colonial morphology of cured strains differs from that of uncured strains

On L agar or NBY agar cured colonies were more flat than uncured colonies, and they appeared whiter than uncured ones. This was true of all the cured strains we tested including those isolated by other procedures. It seems as if the difference in colonial morphology is a reflection of differences in sporulation discussed below. The characteristic morphology of cured colonies served as a very useful indicator to distinguish cured strains from uncured ones.

D. Enhanced sporulation of strains cured of pBA1

Weybridge A cells cured of pBA1 sporulate earlier and at a higher frequency than uncured cells. The involvement of extrachromosomal elements

in sporulation is well-documented. Converting phages are able to induce sporulation in certain asporogenous or oligosporogenous mutants of Bacillus subtilis (5), Bacillus pumilus (6), and Bacillus thuringiensis (7). Loss of a plasmid from an oligosporogenous mutant of B. pumilus greatly increased the frequency of sporulation (8).

In NBY broth at 37°C shaken cultures of Weybridge A mutants cured of pBA1 sporulated considerably earlier than uncured Weybridge A cultures. After 12 hours of incubation 5% of colony-forming-units (CFU) of cured strains were in the form of heat-resistant spores; similar cultures of uncured strains had no heat-resistant CFU after 12 hours. After 18 hours approximately 30% of the CFU of cured cultures were spores, but only about 5% of the CFU of uncured strains were spores. Upon continued incubation the numbers of spores in the uncured cultures approached those in the cured cultures. However, the final extent of sporulation was always higher with cured cells than with uncured cells.

It is difficult to quantitate very precisely the final extent of sporulation of cured cells by the method used above. The problem lies in the fact that asporogenous and oligosporogenous mutants occur at a very high frequency in cultures of cured strains. Therefore, the final number of spores in a culture of cured cells is not always indicative of the true extent of sporulation of cured cells. However, there can be no doubt that cured cells sporulate earlier than uncured cells in broth.

The differences between cured and uncured strains in time and extent of sporulation can be more dramatically demonstrated by phase-contrast microscopic examination of cells from individual colonies. After 18 hours at 37°C colonies of uncured cells of Weybridge A on L agar contained few, if any, spores. However, after the same time colonies of cells cured of pBA1 showed moderate to very extensive sporulation. After 36 hours colonies of uncured cells had only moderate numbers of spores (10 to 20%) and colonies of cured strains had 90 to 100% spores.

E. Heat resistance of spores from cured and uncured strains

We have made the surprising observation that spores from Weybridge A cells cured of pBA1 are more heat resistant than spores from uncured cells. Some data on heat resistance of spores from cured and uncured strains are given in Table 2. After 30 minutes at 85°C, 65% of the spores from

Table 2

Heat resistance of spores of $(pBA1)^+$ and $(pBA1)^-$ strains*

| Strain | % Survival following heating at 85°C for | | |
|---|---|---|---|
| | 30 minutes | 60 minutes | 90 minutes |
| Weybridge A M23 $Ura^-$ (pBA1) | 4 | | 0.0 |
| Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | 65 | | 27.5 |
| Weybridge A M18 *pyrA* (pBA1) | 2 | 0.09 | |
| Weybridge A M18 tdl cured 39 (pBA1, pBC16)$^-$ | 50 | 23.9 | |

*All spore suspensions were previously held at 65°C for 30 minutes to kill vegetative cells.

Weybridge A M23 cured 1 Ura$^-$ (pBA1)$^-$ were still viable but only 4% of the spores from the parental strain, A M23 Ura$^-$ (pBA1) were viable. After 90 minutes these values had decreased to 27.5% and 0.04%, respectively. Similarly, after 30 minutes at 85°C, 50% of the spores from A M18 tdl cured 39 pyrA (pBA1)$^-$ were viable but only 2% of the spores from the parental uncured strain, A M18 pyrA (pBA1), retained viability. After 60 minutes these values had decreased to 23.9% and 0.09%, respectively.

We hope to extend our observations on the differences between cured and uncured strains with respect to their capacity to sporulate and the heat resistance of their spores to determine whether these effects can be attributed specifically to plasmid pBA1 or whether they may be nonspecific effects of plasmids in general. With our experience in CP-51-mediated transduction of plasmids, along with our recent success in transformation of the Weybridge strain, we are in a good position to move other plasmids into cured cells to test their effects, if any, on sporulation.

F. Altered sensitivity of cured strains to bacteriophages

Cells of the Weybridge A strain cured of pBA1 appear to be more sensitive than uncured cells to phages CP-2, CP-20, and CP-51. These three phages were isolated from soil several years ago in my laboratory during a search for phages active on B. cereus. Later tests showed that they are also active on the Weybridge strain of B. anthracis. CP-51 is a generalized transducing phage for B. cereus, B. thuringiensis, and B. anthracis (3, 10, 11) and has been fairly well characterized (12). CP-2 and CP-20 were originally tested for generalized transduction of B. cereus. However, the results were negative and these two phages have not been investigated further.

Data in Table 3 compare the efficiencies of plating of phage CP-51 propagated on cured and uncured strains and assayed on cured and uncured strains. CP-51 grows on both cured and uncured strains. Although in Table 3 the yield of phage grown on uncured cells was only one-tenth of the yield from cured cells, such was not always the case. Yields of CP-51 varied from experiment to experiment and the reason for such variation has not been clarified. Identical conditions were used in Table 3 for growth of phage on the cured and uncured strains. Other lysates from uncured strains have had titers in the order of $10^{10}$ to $10^{11}$ PFU/ml.

Table 3

Efficiencies of plating on various hosts of CP-51 propagated on cured and uncured strains of B. anthracis

| Host for propagation | PFU/ml when assayed on | | | | |
|---|---|---|---|---|---|
| | B. cereus 569 | Weybridge A M23 | | Weybridge A M18 tdl | |
| | | Cured | Uncured | Cured | Uncured |
| Weybridge A M18 tdl pyrA (pBC16, pBA1) | $2.3 \times 10^9$ | $2.1 \times 10^9$ | 0* | $2.3 \times 10^9$ | 0* |
| Weybridge A M18 tdl cured 28 pyrA (pBC16) $(pBA1)^-$ | $4.0 \times 10^{10}$ | $3.7 \times 10^{10}$ | 0* | $3.9 \times 10^{10}$ | 0* |

*Occasionally very faint plaques could be detected on some assay plates but no conditions were found that resulted in formation of distinct plaques on the uncured strains. The same procedure was used for propagation of CP-51 on cured and uncured cells.

It appears that some of the variation in phage yields may be a result of physiological differences in host cells used for the propagation and such differences may be a reflection of uncontrolled variation. At this point it is not clear from our data whether differences in yields of CP-51 can be strictly correlated with the presence or absence of plasmid pBA1. This will be investigated further.

Under the conditions used routinely for plaque assay of CP-51 on B. cereus 569, cured strains of Weybridge A produced very distinct plaques and the efficiency of plating was comparable to that found with B. cereus 569. However, uncured strains generally failed to produce plaques. Occasionally very faint plaques too indistinct to be scored could be detected on some assay plates with uncured cells as the indicator, but usually there was a complete absence of detectable plaques. In spot tests in soft agar for phage activity on uncured cells relatively high concentrations of phage caused a general thinning of the lawn but individual plaques were not discernible.

The data in Table 4 show results with phages CP-2 and CP-20. Both of these phages also grew on uncured cells but the yields were considerably lower than those from cured cells. Phage activity on uncured strains could be demonstrated in spot tests with CP-2 and CP-20 lysates at low dilutions, but dilutions of lysates that produced well defined plaques on lawns of cured cells gave no discernible plaques or lysis on lawns of uncured cells.

To account for the difference in sensitivity of cured and uncured cells to bacteriophages we have thought of the following possiblities: (1) A restriction and modification system in which uncured cells carrying pBA1 would restrict phage grown on cells not harboring the plasmid; (2) Cured cells may adsorb the phages more efficiently than uncured cells, i.e., the presence of pBA1 may result in fewer or altered adsorption sites; (3) pBA1 may interfere with replication of the infecting phage genome; and (4) The difference in phage sensitivity of cured and uncured strains may be a reflection of physiological differences in cured and uncured cells and may be only indirectly related to the presence or absence of pBA1.

We have tentatively concluded that restriction and modification are probably not responsible for the observed differences in sensitivity on the basis of the following observations. The failure of phages to plaque on uncured strains is apparently not related to the host on which

Table 4

Differences in yields of phages CP-2 and CP-20 when propagated on cured and uncured strains of B. anthracis*

| Phage | Host for propagation | PFU/ml when assayed on B. cereus 5464C |
|---|---|---|
| CP-2 | Weybridge A M18 tdl pyrA (pBA1, pBC16) | $7.0 \times 10^5$ |
| CP-2 | Weybridge A M18 tdl cured 28 pyrA (pBC16) $(pBA1)^-$ | $1.0 \times 10^8$ |
| CP-2 | Weybridge A M23 $Ura^-$ (pBA1) | $1.0 \times 10^6$ |
| CP-2 | Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | $1.0 \times 10^8$ |
| CP-20 | Weybridge A M18 tdl pyrA (pBA1, pBC16) | $6.0 \times 10^7$ |
| CP-20 | Weybridge A M18 tdl cured 28 pyrA (pBC16) $(pBA1)^-$ | $1.0 \times 10^9$ |
| CP-20 | Weybridge A M23 $Ura^-$ (pBA1) | $1.0 \times 10^8$ |
| CP-20 | Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | $1.0 \times 10^9$ |

*The cured strains were isolated by the method described in this report. The procedures for propagation of phage on cured and uncured strains were identical.

the phage was grown. In our experiments thus far completed it apparently made no difference whether phage was grown on cured or uncured strains; lysates plaqued on cured but not on uncured strains. This was true of phage CP-51, shown in Table 3, as well as phages CP-2 and CP-20 (data not shown).

It might be predicted that phage CP-51 would not be restricted. This phage contains an odd base, hydroxymethyluracil, instead of uracil (12) and might, therefore, not be sensitive to many restriction endonucleases. However, results of transduction of pBC16 mediated by CP-51 also suggest that a restriction-modification system is not encoded by pBA1. Table 5 presents data from CP-51 transduction experiments with cured and uncured strains. Phage propagated on cells cured of pBA1 but still carrying pBC16 transduced both cured and uncured cells to tetracycline resistance, i.e., $(pBC16)^{+}$. Also phage propagated on uncured strains transduced cured strains. If pBA1 encoded a restriction-modification system, one might expect to obtain significantly fewer transductants with uncured recipients than with cured recipients when the transducing phage was grown on a cured strain. In fact, more transductants were obtained with uncured cells than with cured cells when the donor was A M18 td1 cured 28 and the recipients were A M23 and A M23 cured 1. However, the difference is probably insignificant and not related directly to pBA1. Fewer transductants with cured recipients may be a reflection of the fact that CP-51 is more lytic for cured than for uncured cells. In any case the data do not appear to be consistent with the idea that pBA1 encodes a restriction-modification system.

The second possiblity listed above to account for difference in phage sensitivity of cured and uncured strains, i.e., difference in adsorption, is probably unlikely. Results of preliminary experiments with CP-51 suggest that cured and uncured cells do not differ from each other in their phage adsorption characteristics. However, more definitive experiments on adsorption kinetics will be done before this possibility is definitely ruled out. Also CP-2 and CP-20 will be included in adsorption experiments to be done in the future.

Possibilities 3 and 4 listed above may be difficult to distinguish. Experiments are planned to determine the average burst size of the three phages when grown on cured and uncured cells. If pBA1 interferes with

Table 5

Transfer of pBC16 to cured and uncured strains of B. anthracis by phage CP-51*

| Phage lysate | | Recipient | $Tet^R$ transductants per membrane** |
|---|---|---|---|
| Donor | PFU/ml | | |
| Weybridge A M18 tdl cured 28 *pyrA* (pBC16) $(pBA1)^-$ | $4.2 \times 10^{10}$ | Weybridge A M23 $Ura^-$ (pBA1) | 42 |
| " | " | Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | 23 |
| " | $5.0 \times 10^{10}$ | Weybridge A M23 $Ura^-$ (pBA1) | 54 |
| " | " | Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | 31 |
| Weybridge A M18 tdl *pyrA* (pBA1, pBC16) | $1.0 \times 10^{10}$ | Weybridge A M18 td2 cured 25 $(pBA1, pBC16)^-$ | 39 |
| Weybridge A M2 tdl $Trp^-$ (pBA1, pBC16) | $1.8 \times 10^{10}$ | Weybridge A M23 cured 1 $Ura^-$ $(pBA1)^-$ | 22 |

*Transductions were carried out on Millipore membranes as described under methods.

**Each value represents the average number of transductants from four membranes.

phage replication one would expect a smaller burst size with uncured cells than with cured cells. However, demonstration of a smaller burst size with uncured cells would not necessarily prove that pBA1 was interfering with phage replication. Such a demonstration would, however, imply a rather direct role of pBA1 in decreased sensitivity of cells harboring the plasmid.

The observation that phages CP-2, CP-20, and CP-51 plaque very well on cured cells but extremely poorly or not at all on uncured cells may merely reflect differences in growth characteristics of the two types of cells. The differences between cured and uncured cells in time and frequency of sporulation and in their growth characteristics on synthetic medium might also be related to their differences in capacity to support phage plaque formation. Many factors can affect plaque formation for reasons that are not always obvious. For example I have observed that phage TP-12 will grow very well on wild-type B. thuringiensis 4060C and a number of auxotrophic mutants derived from it. However, it does not form discernible plaques on wild-type 4060C or most 4060C mutants under any conditions I have tested. It does plaque very well on a Trp$^-$ mutant of 4060C which is also oligosporogenous. Very likely the ability of this mutant to support plaque formation is related to its impairment in spore formation. Other Trp$^-$ mutants which are not oligosporogenous do not support plaque formation.

Another interesting observation regarding peculiarities of plaque-forming systems was made in my laboratory with phage CP-54 which is similar in many respects to phage CP-51. CP-54 plaques on many strains of B. thuringiensis on ordinary rich media such as NBY agar or PA agar which are often used for phage assays. However, on two strains, NRRL B-4040 and B-4041, CP-54 plaques were detectable only when a synthetic medium, Minimal 1, was used for the phage assays. CP-54 will grow well on these two strains in rich media, but for some unknown reason plaques are formed only on synthetic media.

We plan to test various factors to see whether we can find conditions that will support plaque formation with CP-2, CP-20, and CP-51 on uncured strains of B. anthracis. Defining conditions that support plaque formation with uncured strains might shed some insight into the nature of the difference between cured and uncured strains with respect to phage sensitivity. Hopefully, such information will also help to assess the role, if any, of pBA1

in suppressing plaque formation.

G. Altered growth characteristics of strains cured of pBA1

Weybridge A mutants cured of plasmid pBA1 exhibit growth characteristics different from those of the parental strain. Cured strains grew considerably more poorly than parental strains on minimal media. We have been investigating this phenomenon in attempts to define the difference in growth requirements. We have used Minimal IC and Minimal M and O in these studies. Most of the studies have been done on agar media but we have also included minimal broth in order to avoid possible contaminating nutrients in agar.

Cured and uncured strains appeared to grow equally well on Minimal IC which contains hydrolyzed casein. Both cured and uncured strains grew faster on Minimal IC than on any completely synthetic medium, e.g., Minimal M and Minimal O, we have tested. However, uncured strains grew significantly better on Minimal M and O than cured strains, both in liquid medium and on agar.

Addition of individual amino acids, purines, pyrimidines, or vitamins did not improve growth of cured strains on Minimal O medium. Addition of multiple amino acids to either medium resulted in improved growth of cured strains. Addition of the three branched chain amino acids, leucine, isoleucine, and valine, to Minimal O definitely resulted in improved growth of cured strains. Colonies of cured strains on Minimal O agar supplemented with these three amino acids were about as large and grew about as fast as colonies of uncured strains on unsupplemented Minimal O agar. However, cured strains did not have an absolute requirement for branched chain amino acids. Similar results were obtained by addition of various combinations of amino acids. When about 10 amino acids were included in Minimal O, the cured strains grew as well as uncured. However, it did not appear to matter which amino acids were included in the mixture.

Thus, it appears that although strains cured of pBA1 have altered growth characteristics in minimal media, it seems very unlikely that loss of the plasmid has resulted in acquisition of specific growth requirements. It seems more likely that loss of the plasmid has affected regulatory activities or perhaps transport activities of the cell. The two observations discussed below are consistent with the idea that the altered growth characteristics of cured strains may be related to altered regulatory or

transport activities rather than a loss of structural genes for synthesis of specific amino acids.

Minimal M and O media contain rather high concentrations of phosphate, 0.04 M $KH_2PO_4$ and 0.08 M $K_2HPO_4$ or a final concentration of 0.12 M phosphate. The growth of cured strains was rather dramatically improved on Minimal O medium when the concentration of phosphate was reduced. A concentration of 0.015 M appeared to be about optimal; further decrease did not improve the growth. However, uncured strains grew better with the original high concentration of phosphate, 0.12 M. On Minimal O medium cured strains grew almost as well with 0.015 M phosphate as uncured strains did with 0.12 M. Conceivably improved growth of cured strains at lower phosphate concentrations could be a reflection of metallic ion requirements. However, we have tested addition of $Ca^{++}$, $Cu^{++}$, and $Zn^{++}$, as well as higher concentrations of $Fe^{+++}$ and $Mn^{++}$ to Minimal O medium without any significant effects on growth.

When cells or spores of cured strains were spread or streaked on Minimal M or O agar, the growth was relatively poor compared to that of uncured strains. However, colonies of "revertants" that grew as well as uncured strains occurred at rather high frequencies. We refer to these as large colony mutants (LCMs). These mutants resemble their original parental uncured strains, not only in their growth characteristics, but also in their sporulation characteristics. They have lost the enchanced sporulation characteristics of their cured parental strains.

It should be made clear that all these studies have been carried out with genetically marked strains so that it is possible to identify variants as arising from a given parental strain and thereby rule out the possibility that they are contaminants. For example, we first isolated and characterized the growth requirements of auxotrophic mutants of our original Weybridge A strain. Certain of these were selected for curing studies and variants cured of pBA1 were shown to have retained the original auxotrophic mutation. Finally, large colony mutants isolated from cured strains were shown, in turn, to have retained the auxotrophic markers of their respective parents.

It occurred to us that "cured" strains which give rise to LCMs might not really be cured of pBA1 but that pBA1 might be integrated into the chromosome. The LCMs might then be formed by excision of the plasmid from the chromosome. However, we have tested some LCMs for presence of plasmid and the ability to produce protective antigen with negative results. It appears more likely that

the LCM phenotype is the result of a mutation which suppresses the effects of loss of pBA1. The nature of such mutation is not at all clear.

H. Current procedure for extracting and demonstrating plasmid pBA1

The best procedure we have found for routine screening of cultures for the presence or absence of pBA1 is the following, which is a modification of the procedure devised by Kado and Liu (13). Other investigators working on B. anthracis might find our modified method useful and for that reason details of the procedure are included here.

Reagents

E buffer

0.04 M Tris base

0.002 M $(Na)_4EDTA$

pH adjusted to 7.9 with glacial acetic acid

E buffer-sucrose

Sucrose (10% w/v) prepared with E buffer

Lysis buffer

0.05 M Tris base

3% (w/v) sodium dodecyl sulfate

pH adjusted to 12.6 with 3 N NaOH

Sucrose (10% w/v) prepared with the buffer

Phenol-chloroform

Freshly distilled phenol mixed with equal volume of chloroform

Pronase

2 mg/ml dissolved in 2 M Tris base previously adjusted to pH 7.0 with 6 N HCl

Tris-borate buffer (10X concentration)

| | |
|---|---|
| Tris base | 108 grams/liter |
| Boric acid | 55 grams/liter |
| $(Na)_2EDTA$ | 9.3 grams/liter |

Agarose

0.7% agarose (Sigma type II) dissolved in tris-borate buffer

Tracking Dye

50% (w/v) glycerol

0.25% bromphenol blue

Prepared in tris-borate buffer

Procedure

Extraction of DNA

Cultures are streaked on L agar plates and incubated overnight at 37°C. A loop of growth from L agar is used to inoculate 25 ml of BHI-glycerol broth in a 250-ml Erlenmeyer flask which is incubated on a shaker (130 rev/min) at 37°C. After 14 to 16 hours 0.3 ml is transferred to 25 ml of the same medium and incubated for 6 hours on the shaker.

Cells from 25 ml of culture are collected by centrifugation in a Sorvall SS-34 rotor at 10,000 rev/min for 10 min at 4°C, and cells are resuspended in 6 ml of E buffer-sucrose. One ml of resuspended cells is transferred to a 1.5 cm x 9 cm polypropylene tube and two ml of lysis buffer are added. The tubes are inverted gently to mix the contents and then held in a 55°C bath for 30 minutes. The tubes are transferred to a 37°C bath and 0.5 ml of pronase is added with gentle mixing. The tubes are removed from the 37°C bath after 20 minutes and 6 ml of cold phenol-chloroform are added. Extraction is effected by inverting the tubes 16 times. Finally the tubes are centrifuged in a Sorvall SS-34 rotor at 10,000 rev/min for 10 min at 4°C. The aqueous layer is removed with a polypropylene transfer pipet.

Electrophoresis

The baby gel electrophoresis unit sold by Bethesda Research Laboratories is most commonly used. The agarose in tris-borate buffer is melted by steaming and cooled to 55°C before pouring. The gel is allowed to harden for at least 40 minutes and then submerged in tris-borate buffer in the electrophoresis unit.

DNA samples are mixed with 5% tracking dye on a Parafilm sheet and then layered into the wells. Electrophoresis is carried out at 50 to 75 volts for 1.5 to 2 hours. Gels are stained with ethidium bromide (1 µg/ml in tris-borate buffer) for 45 minutes. They are destained for at least 30 minutes in distilled water and then examined under UV light.

Comments on the plasmid detection procedure

The main differences in the original method of Kado and Liu and our modification are the pronase treatment and the inclusion of 10% sucrose in the buffers for suspending and lysing the cells. These two additions resulted in more consistent results than were obtained with the original procedure. The age of the cells and the quantity of cells used in the lysis procedure are very critical. We included 0.5% glycerol in the growth medium to prevent sporulation. We had very poor results with cultures that were beginning to sporulate. The use of "baby gels" for the electrophoresis is very convenient and time-saving.

## II. Genetic exchange systems for B. anthracis

### A. Transduction

The Annual Report dated December 1981 describes the use of phage CP-51 to carry out generalized transduction in the Weybridge strain. CP-51 is effective in transducing chromosomal markers and also in transferring a small plasmid, pBC16 (2 megadaltons) among strains of B. anthracis, B. cereus, and B. thuringiensis. Presumably CP-51 will transfer any plasmid small enough to be packaged by the phage but this remains to be seen. We are currently studying the transfer of other small plasmids by CP-51.

We had hoped that CP-51 might be large enough to package and transfer pBA1, but we have been unsuccessful thus far in demonstrating transfer of the plasmid to cured cells of B. anthracis or to cells of B. thuringiensis. We actually looked for cotransfer of pBA1 and pBC16 by selecting for tetracycline resistance (carried by pBC16) and looking among those transductants for acquisition of pBA1. We hoped to take advantage of the difference between cured and uncured strains in colonial morphology to select colonies of cells that had acquired pBA1. However, we were unable to demonstrate cotransduction of the two plasmids. pBA1 is probably too large to be packaged by CP-51. It has been estimated that the size of the CP-51 genome is 56 megadaltons (12). Mikesell, et al. (4) estimated the size of the large plasmid from the Sterne strain to be 110 megadaltons. Thus, if pBA1 of our Weybridge A strain is the same size it is undoubtedly too large to be packaged by CP-51.

Phages TP-13 (7) and TP-12 (Thorne, unpublished), which are transducing

phages for B. thuringiensis, are large enough to package pBA1 but, unfortunately they are not active on B. anthracis. We have spent some effort in looking for host range mutants of these two phages that would be active on the Weybridge strain, but thus far we have found none. We may continue the search for host range mutants since it would be extremely valuable to be able to transfer pBA1 by transduction.

B. Transformation

Until very recently we have been unable to demonstrate any transformation of B. anthracis. We have tried unsuccessfully to induce competence in intact cells for transformation of plasmid and/or chromosomal DNA. However, most of our attempts at transformation have been with protoplasts in procedures similar to that of Chang and Cohen (14) for transforming B. subtilis protoplasts. We have used a variety of procedures for producing protoplasts and for transforming them with pBC16 (tetracycline resistance) or pUB110 (kanamycin resistance) DNA. The problem in transformation of B. anthracis protoplasts seems to reside in the difficulty of regenerating them into cells following exposure to transforming DNA. Recently we have been able to regenerate Weybridge protoplasts and in fact have recovered cells apparently transformed with pUB110 DNA. The frequency is extremely low, but hopefully we will be able to improve the procedure now that we have at least had some success. The details of our procedure will be reported later after we have had time to confirm our results and hopefully improve our methods.

Whether we will be able to transform protoplasts for a plasmid as large as pBA1 remains to be seen. We have tried unsuccessfuly to cotransform B. licheniformis 9945A and B. subtilis 168 with pBA1 and pBC16DNA. It is unfortunate that the transformation of B. anthracis reported by Mikesell, et al. (4) was not done with genetically marked strains. We have tried their procedure but without success even with small plasmids. We always use genetically marked strains in our transformation attempts. Many times we have found colonies which presumably were transformants; however, analysis of their genetic markers showed that they could not be transformants of the particular recipients used.

III. Isolation of auxotrophic mutants and chromosomal mapping

This has been a somewhat neglected area of our proposed research. We have not established any linkages beyond those reported in the Annual Report of December 1981. This neglect has not necessarily been a matter of choice; rather it seemed more profitable to gain a better background on other aspects of B. anthracis genetics and physiology. For example, it seemed very important to learn whether the altered growth characteristics of strains cured of pBA1 were a result of loss of certain structural genes for synthesis of factors required for growth. As reported above, our results suggest that such is not the case and that pBA1 may be involved in regulatory or transport mechanisms.

For isolation of auxotrophic mutants and chromosomal mapping it seems desirable to use a strain or variant that has a minimum of nutritional requirements and is very stable with respect to spontaneous variation. It seems that the large colony mutants derived from strains cured of pBA1 and described above may be desirable for these studies. They grow well on Minimal O medium and thus far appear to be quite stable. Their metabolism is not complicated by the presence of pBA1. We are currently collecting auxotrophic mutants of some of these in preparation for resuming our studies on chromosomal mapping.

IV. Physiological and metabolic factors affecting protective antigen synthesis and accumulation

During the past year we have not done extensive tests on growth conditions affecting protective antigen yields. We have operated under the idea that investigation of B. anthracis genetics is likely to be more fruitful in eventually improving protective antigen yields than extensive tests on arbitrary variations in medium and culture conditions. We have done tests on protective antigen production and confirmed that we can obtain reproducible results routinely and that we have a good system for testing various mutants or variants resulting from genetic manipulation.

Our standard medium for protective antigen production is the Casamino acids medium of Thorne and Belton (1) with bicarbonate and glucose concentrations increased to 0.2% and 0.8%, respectively. Samples are assayed for protective antigen by the agar diffusion method described by Thorne and Belton (1). Maximum yields of protective antigen occur in about

27 hours, resulting in titers in the range of 16 to 24 (reciprocal of highest dilution producing a detectable line of precipitation) when the strength of the antiserum is not the limiting factor.

When Ristroph and Ivins (15) reported their improved medium for toxin production, R medium, we tested it with the idea that we should probably adopt it as our standard. However, in our hands the R medium was not better than the Casamino acids medium we routinely use. We had hoped that the R medium would give a significant increase in protective antigen yields but this was not the case. It seems that the completely synthetic R medium is probably comparable to the completely synthetic 599 medium of Puziss and Wright (16) as modified by Thorne and Belton (1) in 1957 and reported by them to give yields similar to those obtained on the Casamino acids medium. Ristroph and Ivins compared their new R medium with the 1095 medium of Wright, et al. (17). In our hands the 1095 medium has routinely been inferior to the modified 599 medium with respect to production of protective antigen.

V. Abstracts of papers to be presented

The following are abstracts of two papers to be presented at the annual meeting of American Society for Microbiology in March 1983.

CP-51 Mediated Interspecies Transduction of Plasmid pBC16 among Bacillus cereus, Bacillus thuringiensis, and Bacillus anthracis. Robert E. Ruhfel*, N.J. Robillard, and C.B. Thorne.

Bacteriophage CP-51, a generalized transducing phage for Bacillus cereus, B. thuringiensis and B. anthracis, has been demonstrated to mediate transduction of plasmid DNA. B. cereus GP7 harbors the 2.8 Md multicopy tetracycline resistance plasmid, pBC16. CP-51 propagated on GP7 transduced tetracycline resistance to B. thuringiensis and B. anthracis strains at frequencies as high as $2 \times 10^{-6}$ transductants per PFU. Tetracycline-resistant transductants contained a newly acquired plasmid having the same molecular weight as pBC16 when tested by agarose gel electrophoresis. Tetracycline-resistant transductants derived from any of the three species were, in turn, effective donors of pBC16 to recipients of all three species.

Effects of Plasmid Loss on the Physiology of Bacillus anthracis.

Norman J. Robillard*, T.M. Koehler, R. Murray, and C.B. Thorne.

The Weybridge (Sterne) strain of Bacillus anthracis carries a large plasmid, pBA1, which has been shown to be involved in toxin production. Genetically marked strains were cured of the plasmid during growth in the presence of various plasmid curing agents. Cured strains failed to produce detectable levels of the protective antigen component of toxin as determined by immunodiffusion. In addition, cured strains exhibited an altered colonial morphology, a marked increase in rate and frequency of sporulation, altered growth characteristics in synthetic media, and increased sensitivity to Bacillus cereus phages CP-2, CP-20, and CP-51. Preliminary experiments suggest that the change in sensitivity to bacteriophages is not a result of restriction and modification system encoded by the plasmid. The altered colonial morphology may be a reflection of the change in sporulation. These two characteristics have also been useful in devising a more efficient procedure for isolating cured strains. By selecting for sporulation at 42°C and screening colonies on the basis of their morphology, we have been able to isolate cured strains at high frequency.

## LITERATURE CITED


1. Thorne, C.B., and F.C. Belton. An agar diffusion method for titrating Bacillus anthracis immunizing antigen and its application to a study of antigen production. J. Gen. Microbiol. 17:505-516(1957).

2. Sterne, M. Variation in Bacillus anthracis. II. Some correlations between colony variation and pathogenicity in strains of Bacillus anthracis. Onderstepoort. J. Vet. Sci. Animal Ind. 8:279-349(1937).

3. Thorne, C.B. Transducing bacteriophage for Bacillus cereus. J. Virol. 2:657-662(1968).

4. Mikesell, P., B.E. Ivins, J.D. Ristroph, and T.M. Drier. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Infect. Immun. 39:371-376(1983).

5. Bramucci, M.G., K.M. Keggins, and P.S. Lovett. Bacteriophage PMB12 conversion of the sporulation defect in RNA polymerase mutants of Bacillus subtilis. J. Virol. 24:194-200(1977).

6. Bramucci, M.G., K.M. Keggins, and P.S. Lovett. Bacteriophage conversion of spore-negative mutants to spore-positive in Bacillus pumilus. J. Virol. 22:194-202(1977).

7. Perlak, F.J., C.L. Mendelsohn, and C.B. Thorne. Converting bacteriophage for sporulation and crystal formation in Bacillus thuringiensis. J. Bacteriol. 140:699-706(1979).

8. Lovett, P.S. Plasmid in Bacillus pumilus and the enhanced sporulation of plasmid-negative variants. J. Bacteriol. 115:291-298(1973).

9. Bernhard, K., H. Schrempf, and W. Goebel. Bacteriocin and antibiotic resistance plasmids in Bacillus cereus and Bacillus subtilis. J. Bacteriol. 133:897-903(1978).

10. Thorne, C.B. Transduction in Bacillus thuringiensis. Appl. Environ. Microbiol. 35:1109-1115(1978).

11. Thorne, C.B. Transduction in Bacillus cereus and Bacillus anthracis. Bacteriol. Rev. 32:358-361(1968).

12. Yelton, D.B. and C.B. Thorne. Comparison of Bacillus cereus bacteriophages CP-51 and CP-53. J. Virol. 8:242-253(1971).

13. Kado, C.I., and S.T. Liu. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373(1981).

14. Chang, S., and S.N. Cohen. High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. Mol. Gen. Genet. 168:111-115 (1979).

15. Ristroph, J.D., and B.E. Ivins. Elaboration of Bacillus anthracis antigens in a new, defined culture medium. Infect. Immun. 39:483-486(1983).

16. Puziss, M., and G.G. Wright. Studies on immunity in anthrax. IV. Factors influencing elaboration of the protective antigen of Bacillus anthracis in chemically defined media. J. Bacteriol. 68:474-482(1954).

17. Wright, G.G., M. Puziss, and W.B. Neely. Studies on immunity in anthrax. IX. Effect of variations in cultural conditions on elaboration of protective antigen by strains of Bacillus anthracis. J. Bacteriol. 83:515-522(1962).

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